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13. ABSTRACT (Maximum 200 Words)

Towards the goal of molecular diagnosis and staging of NF1-related tumors, we proposed to develop and validate a novel PCR-based method, termed QuEST, of rapid and quantitative identification of loci with increases of decreases in genomic copy number in MPNST. We have developed new assays that are sensitive and specific and allow the quantification of one gene copy versus two gene copies. The method was validated on constitutional DNA using assays that determined the copy number of the NF1 gene. This methodology will be used for both loss (deletion) of DNA and gain (amplifications) of DNA in tumor cell lines and primary tumors. Assays are in development for the target areas of chromosome 1p and 17q22-24. The amplification and loss of chromosomal material at these regions was confirmed by high resolution karyotypingin two neuroblastoma-derived cell lines that will be emplyed to test the methodology on tumor tissues. We have made significant progress in assay design for quantification of genomic losses and gains. Implementation of these assays will now test the usefulness of our idea of using QuEST for defining specific losses and gains in tumors.

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Introduction

Lack of a definitive system for the diagnosis and staging of NF1-related tumors is a major obstacle to investigating the molecular basis of tumorigenesis, to our ability to assess prognosis, and consequently, to the rational design and application of stage-specific therapeutic agents (1-3). Locus-specific changes in copy number are a common feature of many types of tumors, including the neurofibromas and malignant peripheral nerve sheath (MPNST) that develop in individuals with NF1 (4-13). Gains or losses at a particular locus are potential biomarkers for the molecular diagnosis and grading of MPNST. Towards the goal of molecular diagnosis and staging of NF1-related tumors, we propose to develop and validate a novel PCR-based method, termed QuEST, for rapid and quantitative identification of loci with increases or decreases in genomic copy number in MPNST. The specific aims are (1) to develop a series of reference and target markers for quantitative assessment of gene copy number. This will involve selection, design and optimization of primers and QuEST reactions for reference and target loci. (2) to perform QuEST analysis on series of cell lines and MPNST samples for two target regions where copy number changes are associated with MPNST formation: amplification of 17q22-qter and deletion of 1p. (3) to determine the feasibility of coupling QuEST analysis with laser capture microdissection. This study has the potential of providing direct and precise methods to use in addressing issues important to diagnosis, treatment, and molecular analysis of MPNST.

Body

Research accomplishments associated with each task outlined in the Statement of Work are detailed below. We have obtained a one year no-cost extension to complete this project, because our timeline was significantly delayed due to the departure of a postdoctoral fellow who was working on this project. A new postdoctoral fellow (Dr. Wang) is now focusing on the development, optimization, and validation of assays and a new Research Associate (Ms. Vong) is implementing those assays on appropriate samples. With these two new professionals, we anticipate accelerating the timeline to complete the project within the next year. We made significant changes in the methodology of quantitative PCR to increase the sensitivity and precision.

Approved Statement of Work

Task 1. Design and test a series of primers for analysis of both target loci and reference loci.

a. Design and synthesize primers for genes in the target regions of chromosomes 1p and 17q22-24 and for reference loci.
b. Optimize the amplification reaction for each locus using non-tumor genomic DNA

Progress: We need the capability and sensitivity to determine if a locus or gene has no deletion (2 copies), one gene deletion (1 copy), two gene deletion (0 copies), or amplification (likely >10 copies). Two gene deletion and gene amplification are simple to detect using various methods of quantitative PCR. The difficulty is in reliably differentiating one versus two copies of a gene. Therefore, we have been focusing on developing the best assay to detect a one gene deletion. To determine which quantitative PCR methods were sensitive, we choose to develop an assay at intron 31 of the NF1 gene. To validate the assays we used DNA from normal individuals (2 NF1 genes) and DNA from NF1 patients with deletions involving the NF1 gene (1 NF1 gene). Initially, we tried using SYBR green (binds double stranded DNA) fluorescence as a method of detection during real-time PCR in the LightCycler instrument (Roche). There was considerable overlap between the crossing point values (Ct) for samples with one and two NF1 genes indicating that the assay was not as sensitive or specific as required (data not show). Secondly, we sought to increase specificity and sensitivity by using a NF1-specific fluorescently-labeled primer. We constructed a LUX primer (Invitrogen), which

is a hybrid primer comprised of NF1 specific sequences and anonymous sequences that are capable of fold-back annealing. LUX primers are touted as having high specificity because they only fluoresce at high temperatures when the fold backs are melted. We had multiple problems with LUX primers and decided to abandon that approach (data not shown). The third method we developed was precise, sensitive, and specific and involves SYBR green for detection, competitive quantitative PCR, and melting curve analysis as detailed below.

We chose to employ SYBR green for detection in combination with competitive PCR, which is the most suitable method of quantification when highly accurate determinations are required. We adapted and modified a method published by Ruiz-Ponte et al. (14). In this method, a known copy number of a competitor is introduced directly in the PCR mixture along with the target DNA of the patient/tumor. The competitor, which is almost identical to the target DNA but distinguishable by product length, is amplified with the same set of primers so that efficiency of amplification for the two amplicons is the same. Calibration curves of different competitor concentrations determines the optimal concentration that equals that of the target DNA. Figure 1 shows the melting curves of a normal control DNA samples (2 copies of NF1), where competitor and intron 31 are co-amplified with equal efficiency and the area under the curves are equal (roughly equivalent to peak height in this example). We constructed the competitor such that it would be amplified with the intron 31 primers, but have a different melting curve by replacing an internal TTT sequence with a CCC sequence. As expected, negative samples lacking human DNA did not amplify.

Figure 2 shows the melting curve of a patient with an NF1 deletion (1 copy of NF1) versus that of the normal control individual. Note that the amplitude of the melting curve of the NF1 amplicon for the deletion patient is less that of the competitor amplicon because there are fewer targets in the deleted patient's DNA. For precision, we use the peak areas of each melting curves for quantitation rather than peak height. A ratio of peak area of normal control (2 copies) over the peak are of the patient target DNA is calculated, see below.

(see figures on next page)

Figure 1. Melting curve analysis after competitive, quantitative PCR at NF1 intron 31 in genomic DNA of a normal control individual. The peaks representing the melting curve of the amplicon of the competitor and the amplicon of the patient's target DNA are indicated. The negative control without DNA shows evidence of amplificiation.

Figure 2. Melting curve analysis after competitive, quantitative PCR at NF1 intron 31 can differentiate one gene copy versus two gene copies. The results of two reactions are shown, closed circles represent target DNA from a normal control individual and closed squares represent target DNA from an NF1 patient with a deletion of one gene. The peaks representing the melting curve of the amplicon of the competitor and the amplicon of the patient's target DNA are indicated. The negative control without DNA shows evidence of amplification.

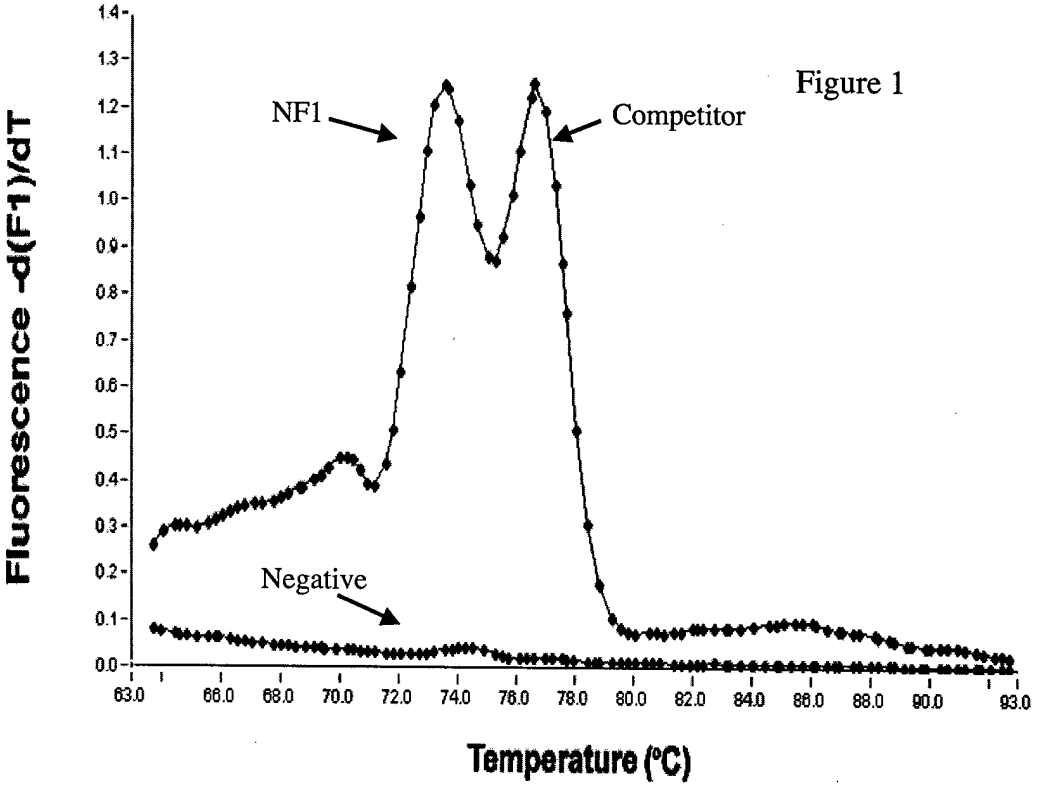


Figure 1

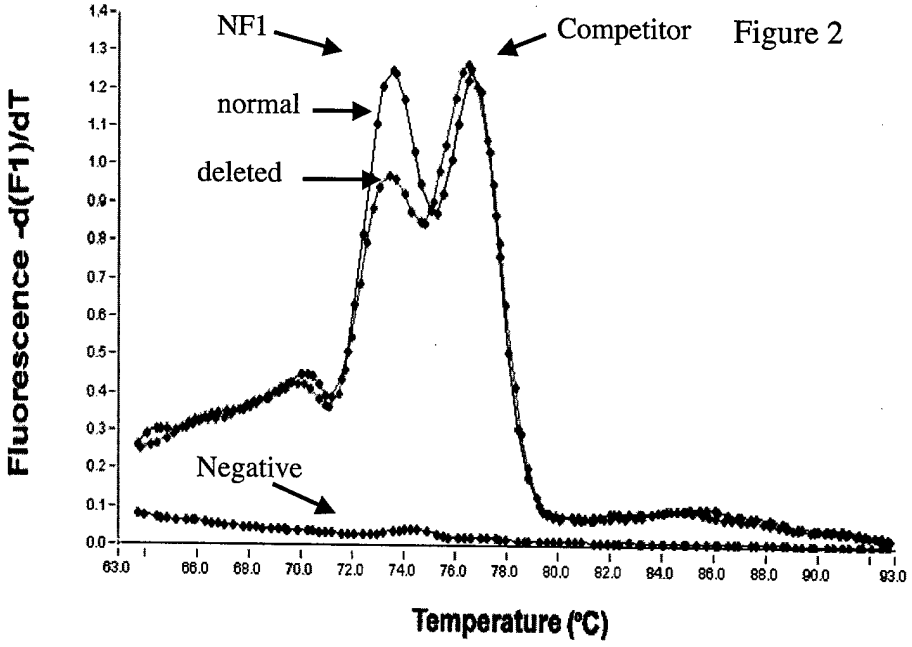


Figure 2

Once the concentration of competitor is determined for a certain concentration of the normal control DNA, it is essential that all subsequent reactions with unknown patient DNA samples contain exactly the same concentration of target DNA. Prior to the competitive quantitative PCR assay, we determine the exact concentration of each patient sample using real-time quantitative PCR at a different locus. We amplify the TPA (tissue plasminogen activator) gene on chromosome 12 in each patient and compare that to a standard curve using the normal control DNA. From this reaction, we can calculate exactly what volume of patient DNA must be added to the competitive quantitative PCR assay. An example of the TPA real-time PCR and standard curve is shown in Figure 3.

(see figure 3 on next page)

Figure 3. LightCycler real-time PCR at TPA locus showing standard curve. The upper panel shows the results of real-time PCR of the TPA locus of a dilution series of a normal control individual. The reaction consists of unlabeled primers and uses an internal labeled (fluorescence resonance energy transfer (FRET) probe for detection of product. The crossing point (Ct) is defined as the fractional cycle at which fluorescence begins to increase exponentially and is calculated by the LightCycler. Ct becomes larger as the number of TPA targets decreases. The lower panel shows the standard curve calculated from the above data. Note the low error and high correlation coefficient.

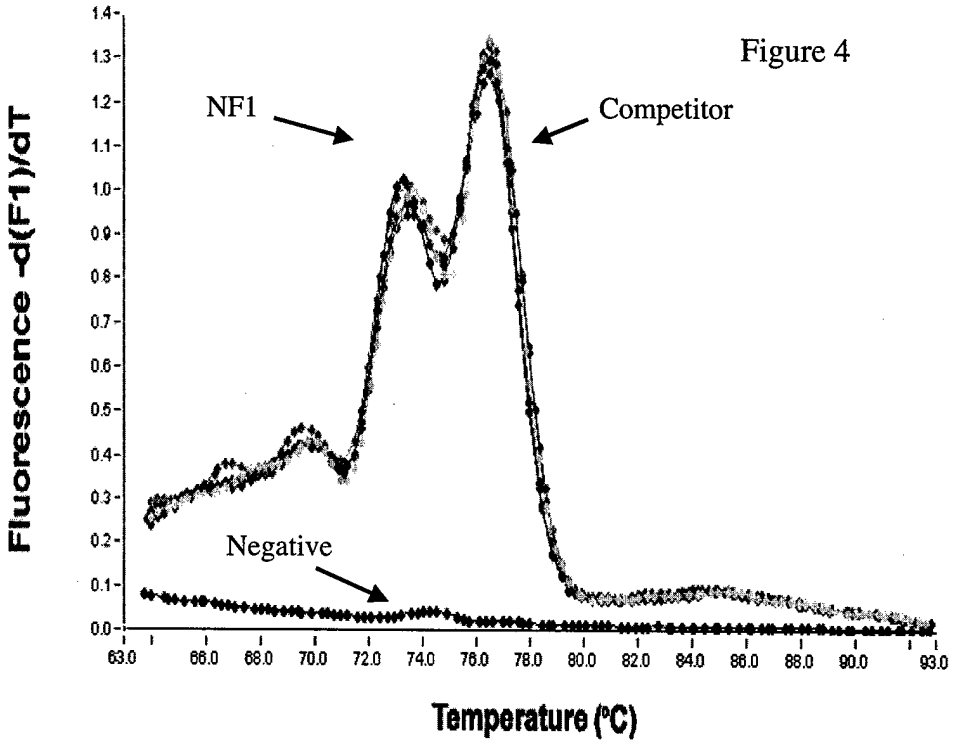


Figure 4

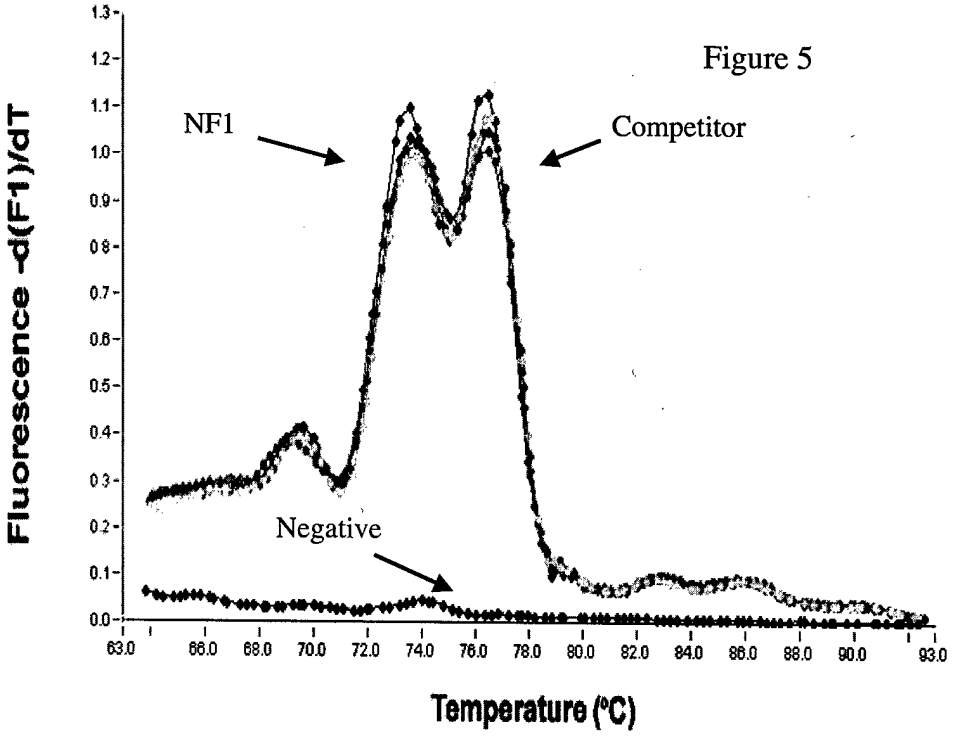


Figure 5

The competitive quantitative gene dosage assay we have developed for intron 31 of the NF1 gene is very precise and shows minimal variability. Figures 4 and 5 (see legends below and figures on next page) show melting curves for 6 replicates of an NF1 deletion patient and a normal control individual, respectively. The data will be analyzed as shown in the tables below. First, note the precision of the assay; the standard deviations are only 2% and 3% of the means of the assay for normal control individuals and deletion patients, respectively. As expected the ratio of NF1:competitor peak areas is essential 1.0 for samples with two copies of the NF1 gene and is 0.5 for samples with one copy of the NF1 gene. We have screened a large collection of NF1 deletion patients and normal individuals and found that there is no overlap of ratio values between these two populations.

Therefore, we have developed a sensitive and specific assay that differentiates realiably between one versus two copies of a gene. We are now applying this same assay to determine it's ability to quantitate amplified loci.

Replicates of competitive quantitative PCR for intron 33 of NF1 or normal control individual

No.	Area of *NF1* peak	Area of Competitor Peak	Ratio	Mean Ratio	S.D.
1	3.219	2.957	1.088		
2	3.262	3.296	0.989		
3	3.097	3.255	0.951		
4	3.464	3.26	1.062		
5	3.433	3.278	1.047		
6	3.091	2.918	1.059		
				1.033	0.0211

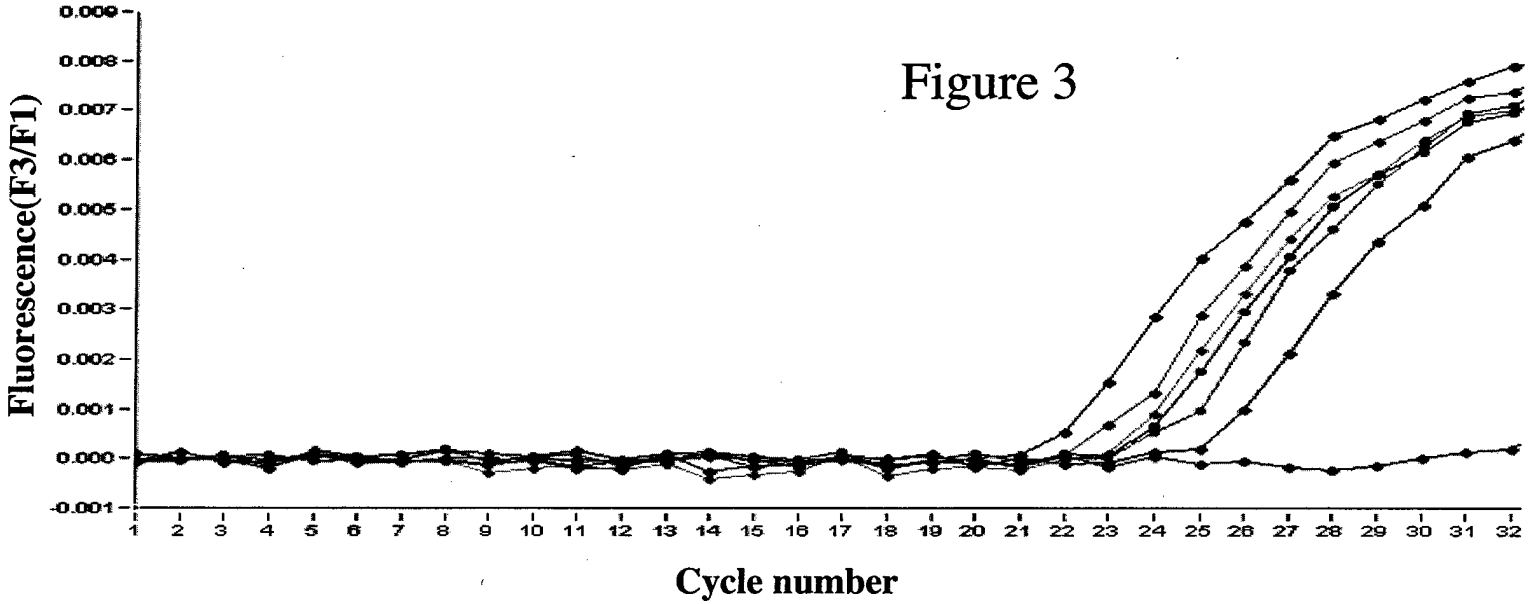
Replicates of competitive quantitative PCR for intron 33 of NF1 for deleted patient

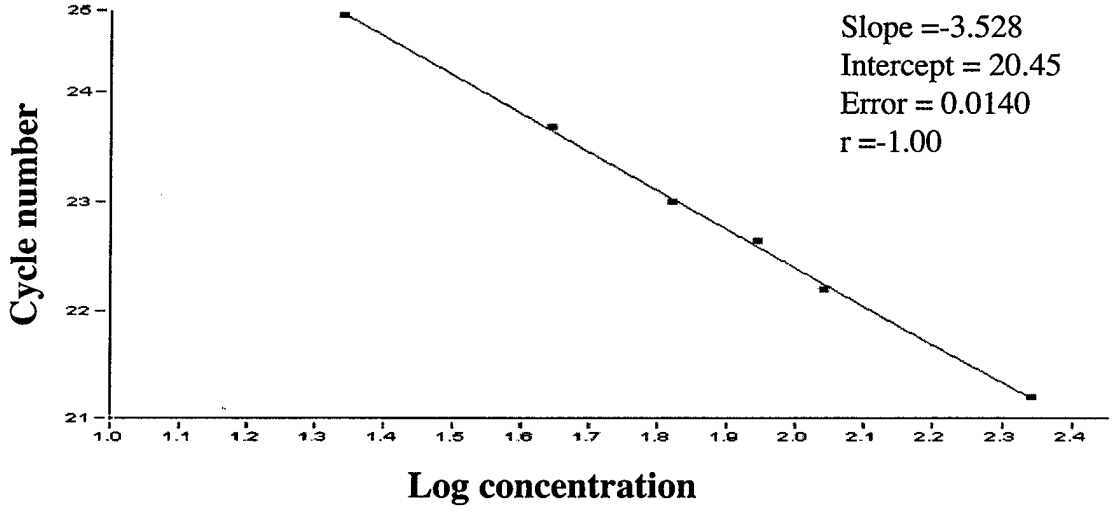
No.	Area of *NF1* peak	Area of Competitor Peak	Ratio	Mean Ratio	S.D.
1	1.937	3.85	0.503		
2	2.103	3.68	0.571		
3	1.952	3.755	0.519		
4	2.052	3.356	0.611		
5	1.851	3.665	0.505		
6	1.934	3.33	0.580		
				0.548	0.0185

Figure 4. Melting curves of competitive quantitative PCR at NF1 intron 31 of 6 replicates of an NF1 deletion patient. Six curves (color replaced by grey tones for this report) are overlaid to demonstrate the precision and lack of variability in replicate samples.

Figure 5. Melting curves of competitive quantitative PCR at NF1 intron 31 of 6 replicates of a normal control individual. Six curves (color replaced by grey tones for this report) are overlaid to demonstrate the precision and lack of variability in replicate samples.

Figure 3


Fluorescence(F3/F1)
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
-0.001
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
Cycle number


Cycle number
26
24
23
22
21
1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9 2.0 2.1 2.2 2.3 2.4
Log concentration
Slope =-3.528
Intercept = 20.45
Error = 0.0140
r =-1.00

Task 2. Perform spectral karyotyping (SKY) of the MPNST- and neuroblastoma-derived cell lines. Months 1-12.

Progress: In collaboration with Dr. Karen Swisshelm, Department of Pathology, University of Washington, we have analyzed the karyotype of two of the neuroblastoma-derived cell lines. We have performed a routine metaphase G-banded karyotype, which was very informative in determining exactly which bands were deleted and or amplified. One line will be ideal for analysis of the amplified region of chromosome 1p and the other line for the deleted region of 17q22.24. Other regions of amplification and deletion were observed and may be targeted later in this project. Because this analysis was so informative, we do not plan to perform a SKY analysis unless our PCR results are inconsistent with the karyotype.

Dr. Stephens performed a comprehensive literature search and compilation of the genetic changes in NF1-associated peripheral nerve sheath tumors (see manuscript in Appendix). This review revealed that there are consistent regions of the genome that undergo gains and losses in MPNST. This review also underscored our choice of the 17q22-q25 region as one of frequent gains in chromosomal loci (74%).

Task 3. Validate the QuEST method by analysis of target and reference loci in DNA from tumor cell lines. Months 6-12.

a. Perform QuEST analysis of genes in the target regions chromosomes 1p and 17q22-24 in DNA from MPNST- and neuroblastoma-derived cell lines. We anticipate analysis of 4 lines.
b. Evaluate the results of QuEST compared to the SKY analysis.

Progress: This analysis is in progress. Now that the competitive quantitative PCR methodology has been validated, we are developing and optimizing similar assays for loci that span the 1p and 17q22-24 regions. Development and ptimization of each assays includes:

- Choosing primer pairs for loci that are single-copy in the genome.
- Screening against the appropriate monochromosomal human-rodent hybrid cell line to ensure we are not amplifying related loci from other chromosomes
- Optimizing PCR reaction
- Developing competitor target DNA
- Titrating competitor target DNA to determine the concentration equivalent to the normal diploid DNA control target
- Performing real-time PCR at TPA locus for each tumor DNA sample (in reality, we will have 5 such loci as controls in case one of them is altered in the tumor sample). Determine the concentration to add to the competitive quantitative PCR assay.
- Performing competitive, quantitative PCR assay
- Analyzing data

As stated in the original Statement of Work, tasks 4-6 will be performed in the later period of the research project.

Task 4. Test the QuEST method by analysis of target and reference loci in DNA from tumors.
This awaits completion of the assays in the tumor cell lines. Anonyous MPNST DNA from NF1 patients are being collected from collaborators for these future assays.

Task 5. Determine the sensitivity of the QuEST method.
This await completion of Task 4.

Task 6. Couple QuEST with laser capture microdissection of archival MPNST.
After Dr. Wang has the assays developed and they are being implemented by Ms. Vong, he will begin performing laser capture microdissection of archival MPNST at the University of Washington Medical Center and assessing the quality of the DNA using the TPA assay. When this protocol is optimized, we will apply the assays for deletion of 17q22-q25 region in MPNST.

Key Research Accomplishments

- A comprehensive analysis and summary of genetic changes reported in NF1-associated peripheral nerve sheath tumors. These data were reported in a review article by Dr. Stephens that is in press (see Reportable Outcomes).
- Developed a specific and sensitive competitive, quantitative PCR methodology that can differentiate one copy from two copies in a genome.
- Employed real-time PCR of the TPA locus as a means of precise DNA quantification
- Validated the competitive, quantitative PCR methodology by assay of the NF1 locus in normal control individuals and NF1 deletion patients.
- Documented the reproducibility of the competitive, quantitative PCR method.
- Performed and analyzed high-resolution karyotype of the two neuroblastoma-derived cell lines that we will be using to validate the competitive, quantitative PCR method for analysis of DNA gains and losses in tumor tissue.

Reportable Outcomes

- Manuscript: Stephens K. Genetics of Neurofibromatosis 1-associated peripheral nerve sheath tumors. Cancer Invest, in press. (See proofs of article in appendix).
- Karyotypes of two neuroblastoma-derived cell lines.
- Development of competitive, quantitative PCR assays for differentiating one versus two copies of NF1 gene intron 31.

Conclusions

Towards the goal of molecular diagnosis and staging of NF1-related tumors, we proposed to develop and validate a novel PCR-based method, termed QuEST, for rapid and quantitative identification of loci with increases or decreases in genomic copy number in MPNST. We have performed a comprehensive literature search and compilation of the genetic changes in NF1-associated peripheral nerve sheath tumors. The implications of this analysis are that consistent regions of the genome frequently undergo gains and losses in NF1-associated MPNST. This phenomenon underscores the validity of our approach and increases the probability that it may be of clinical and/or research use. We have developed new assays that are sensitive and specific and allow the quantitation of one gene copy versus two gene copies. The method was validated

on constitutional DNA using assays that determined the copy number of the NF1 gene. This methodology will be used for both loss (deletion) of DNA and gain (amplifications) of DNA in tumor cell lines and primary tumors. Assays are in development for the target areas of chromosome 1p and 17q22-24. The amplification and loss of chromosomal material at these regions was confirmed by high resolution karyotyping in two neuroblastoma-derived cell lines that will be employed to test the methodology on tumor tissues. We have made significant progress in assay design for quantification of genomic losses and gains. Implementation of these assays will now test the usefulness of our idea of using QuEST for defining specific losses and gains in tumors.

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MOLECULAR BIOLOGY AND GENETICS OF CANCER

Genetics of Neurofibromatosis 1-Associated Peripheral Nerve Sheath Tumors

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AQ1

INTRODUCTION

Much of our current understanding of tumorigenesis is founded on genetic studies of relatively rare individuals with inherited disorders that predispose to certain cancers, e.g., retinoblastoma[1] and colorectal tumors.[2,3] Such genetic studies are consistent with a model whereby normal tissues become highly malignant due to successive mutation of multiple genes that dysregulate cellular proliferation and homeostasis. Important classes of mutated genes include oncogenes (positive growth regulators), tumor suppressors (negative growth regulators), and those encoding cell cycle regulators, antiapototic signals, and components of the DNA replication and repair machinery. Mutation during tumorigenesis can occur at the nucleotide level as inactivation of a single gene or at the chromosomal level as losses of large segments or entire chromosomes, as a fusion of two different chromosomal segments, or as a high level amplification of a segment.[4] Screening normal and tumor tissues of patients for common genetic alterations has been a productive strategy for identifying genes that contribute to tumor formation. This article focuses on genetic changes commonly associated with peripheral nerve sheath tumors in individuals with the autosomal dominant tumor-prone disorder neurofibromatosis 1 (NF1).

Virtually all individuals affected with NF1 develop multiple peripheral nerve sheath tumors. The most common are neurofibromas, which are benign tumors that can occur anywhere along the length of epidermal, dermal, deep peripheral (including dorsal nerve roots in the paraspinal area), or cranial nerves. They do not occur in the brain or the spinal cord proper.[5] Although benign, neurofibromas can cause considerable morbidity by, e.g., infiltrating and functionally impairing normal tissues, causing limb hypertrophy, or masking an emerging malignancy. Some types of neurofibromas can transform into malignant peripheral nerve sheath tumors (MPNST), previously termed neurofibrosarcoma or malignant schwannoma. An estimated 20%–50% of MPNSTs are associated with NF1 disease,[6] and they are a significant cause of the decreased life expectancy in the NF1 patient population.[7,8] Recent population-based longitudinal studies detected an annual incidence of 1.6/1000 and a lifetime risk of 8%–13% for NF1-associated MPNST,[9] which is much higher than detected in previous cross-sectional studies[10] and over three orders of magnitude greater than that of the general population (~0.001%).[11] Furthermore, NF1-associated MPNST were diagnosed at an earlier age than sporadic tumors (26 vs. 62 years, $p < 0.001$) and associated with a poorer prognosis than sporadic MPNST (5-year survival of 21 vs. 42%, $p = 0.09$).[9]

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This article reviews the genetic abnormalities that have been identified at the level of the gene, chromosome, and genome in NF1-associated neurofibromas and MPNST. Identifying commonly associated genetic alterations and the mechanisms by which they arise may potentially lead to markers for tumor staging, to new research approaches to pathogenesis, and to the identification of gene targets, which in conjunction with NF1, will be useful for mouse models.

THE MOLECULAR BASIS OF NF1

The NF1 is a common autosomal dominant disorder that affects about 1 in 3500 individuals worldwide. About 30%–50% of cases are sporadic, caused by de novo mutation in the *NF1* gene of an individual without a family history of the disorder. In addition to predisposing to tumorigenesis, NF1 is associated with characteristic changes in pigmentation and can be associated with a wide range of other manifestations such as learning disabilities and bony abnormalities (reviewed by Refs. [12–16]). All cases are caused by mutation of the *NF1* gene at chromosome 17 band q11.2, which contains 60 exons that encode the 2818 amino acid protein called neurofibromin.[17–21] Neurofibromin is widely expressed, predominantly in the central nervous system and sensory neurons and Schwann cells of the peripheral nervous system.[22,23] One functional domain of neurofibromin has been defined, the GAP-related domain (GRD), so called because of its structural and functional homology to mammalian p120-GAP (*G*TPase *a*ctivating *p*rotein) and yeast genes known to regulate the Ras pathway.[24,25] The Ras-GAP proteins function as negative regulators of Ras by catalyzing the conversion of active GTP-bound Ras to the inactive GDP-bound Ras form.[26] In NF1-associated peripheral nerve sheath tumors, it is hypothesized that neurofibromin deficiency leads to increased activated Ras, resulting in aberrant mitogenic signaling and the consequent growth of a tumor. The identification of an NF1 patient with a missense mutation in the GRD that specifically abolished the Ras-GAP activity of neurofibromin demonstrated the importance of neurofibromin GAP function in NF1 pathogenesis.[27] In at least some tissues, there is evidence that it is the Ras-GAP activity that accounts for the tumor suppressor function of neurofibromin. The most complete evidence comes from genetic and biochemical analyses of NF1-associated malignant myeloid disorders.[28,29] The tumor suppressor function of *NF1* has been reviewed recently[30–32] along with its potential as a therapeutic target.[33]

The NF1 disease is caused by haploinsufficiency for neurofibromin. The vast majority of *NF1* mutations are not in the GRD but are distributed throughout the gene. Over 80% of mutations inactivate or predict inactivation of neurofibromin; splicing defects and sequence alterations that create a premature translation termination codon are the most common.[34,35] About 10% of *NF1* mutations are missense,[35,36] which are typically clustered in the GRD or an upstream cysteine/serine-rich domain that may play a role in ATP binding.[36] Definitive evidence that neurofibromin haploinsufficiency underlies NF1 came with the identification of *NF1* whole gene deletions in an estimated 5%–10% of affected individuals.[37,38] Mutational analyses of patients with specific features, such as plexiform neurofibroma,[39] spinal neurofibroma,[40] or malignant myeloid disorders,[41] failed to detect any correlation between genotype and phenotype. A notable exception is the subset of patients heterozygous for a microdeletion spanning the entire *NF1* gene, who consistently show an early age at onset of cutaneous neurofibromas (see below).

Virtually all NF1 patients develop neurofibromas. Neurofibromas are comprised of an admixture of largely Schwann cells and fibroblasts, along with mast cells, endothelial cells, and pericytes.[42,43] Although classification schemes vary, Friedman and Riccardi[13] define four types of neurofibromas. Discrete cutaneous neurofibromas of the epidermis or dermis are the most common, typically appearing near or at puberty and increasing in number to over 100 by the fourth decade of life.[44] They are a localized tumor of small nerves in the skin that feels fleshy and soft; they are more prevalent on the trunk but also occur frequently on the face and extremities. Discrete subcutaneous neurofibromas have a spherical or ovoid shape, feel firm or rubbery, and may be painful or tender. Deep nodular neurofibromas, also called nodular plexiform neurofibromas, involve major or minor nerves in tissues beneath the dermis. On gross pathology, they appear to grow inside the peripheral nerve, causing a fusiform enlargement, and may extend the entire length of a nerve.[43] Diffuse plexiform neurofibromas have a cellular composition similar to that of cutaneous neurofibromas, but in contrast, they have a tendency to become locally invasive.[5,43] Histologically, this tumor is a tangled network involving multiple nerve fascicles or branches of major nerves with poorly defined margins that makes complete surgical resection virtually impossible. Plexiform neurofibromas can be superficial with extensive involvement of underlying tissues, or they may involve deep tissues, particularly in the craniofacial region, paraspinal structures, retroperitoneum, and gastrointestinal tract.[13] They can infiltrate soft

tissues resulting in localized hypertrophy and significant functional impairment. In contrast to other types of neurofibromas, diffuse plexiform tumors are considered congenital because they typically become evident in infancy or childhood and rarely, if ever, in late adulthood.[45,46] In a population-based study, 32% of individuals with NF1 had a plexiform neurofibroma(s) on physical examination.[47] Korf[5] recently reviewed plexiform neurofibromas.

Plexiform neurofibromas, both diffuse and nodular, are at a greater risk of transforming to an MPNST than other types of neurofibromas (reviewed in Refs. [5,43,48]). Pathological examination of MPNST from individuals, both with and without NF1, most often shows an association with a neurofibroma.[11,49–51] These data suggest that an early step in MPNST development may be preneoplastic process in the nerve sheath. Multiple pathological and molecular criteria are used to evaluate a neurofibroma for malignant transformation.[48] Several lines of evidence are consistent with cutaneous and plexiform neurofibromas and MPNSTs being clonal tumors that arise from an ancestral Schwann cell (see below).

GENETICS OF NEUROFIBROMAGENESIS

Homozygous Inactivation of *NF1*

Homozygous inactivation of a tumor suppressor gene(s) is a fundamental mechanism of tumorigenesis. It occurs by either sequential somatic inactivation of both alleles or by a somatic mutation in the single normal homolog in individuals who inherit a germline mutation in one allele. Somatic inactivation is frequently associated with loss of heterozygosity (LOH) at the tumor suppressor locus and at multiple flanking loci (reviewed in Refs. [52,53]). Evidence for such "2nd hit" somatic *NF1* mutations in neurofibromas has been sought in support of the hypothesis that neurofibromin functions as a tumor suppressor in Schwann cells. Initial reports of LOH at *NF1*[54] have been confirmed and extended by analyses of both primary tumor tissue and neurofibroma-derived Schwann cells. At least 25% of neurofibromas undergo LOH at *NF1*.[55,56] The cellular admixture in neurofibromas can mask allelic loss,[55,56] which is the likely explanation for reports that detected few, if any, tumors with LOH.[57–62] Compelling evidence that the somatic inactivation of the *NF1* gene itself is important was provided by the identification of a 4 bp deletion in exon 4b in a cutaneous neurofibroma of a patient with a germline *NF1* microdeletion.[63] Subsequent analyses of cDNA from neurofibromin-derived Schwann cells showed that 19% of neurofibromas carried somatic intragenic mutations, which were typically mRNA splicing defects.[34] Mutation frequency may be underrepresented due to the difficulty of recovering high-quality tumor RNA and the underrepresentation of mutant transcripts observed in some tumors. The latter could be attributed to mutations that induce nonsense-mediated decay or other mechanisms that affect mRNA content (reviewed in Ref. [64]) or to reduced expression for other reasons,[34] and/or a low proportion of mutant to normal Schwann cells in some tumors.[65,66] Homozygous inactivation of *NF1* also occurs in plexiform neurofibromas, where an estimated 40% (n = 10) showed LOH,[67] a result confirmed by subsequent reports.[57,58,68,69]

A predominant mechanism of somatic *NF1* inactivation in neurofibromas that underwent LOH was mitotic recombination.[56] A 17q proximal single mitotic recombination event near the centromere of the q arm between the normal chromosome 17 and the homolog carrying the germline *NF1* mutation can generate a cell in which both *NF1* genes carry the germline mutation and all loci distal to the recombination site are identical. Less common were double recombination events that result in chromosome 17 interstitial loci showing LOH. The *NF1* mRNA in some neurofibromas is edited such that an arginine codon is changed to a nonsense codon.[70] Although only one-third of neurofibromas examined showed a low level (<2.5%) of edited *NF1* transcripts,[71] such modulation of neurofibromin expression may be important if, e.g., editing occurred at high frequency in transcripts from a specific minor cellular component of the tumor. In other neurofibromas, somatic mutations appear to destabilize *NF1* mRNA.[66] Transcriptional silencing via hypermethylation of promoter regions, a prominent mechanism of inactivating other tumor suppressor genes,[72] has not been detected in neurofibroma tissue.[73,74] As expected from the mutational and LOH analyses, some neurofibromas had no detectable *NF1* transcripts or neurofibromin.[75,76] A quantitative Ras activity assay demonstrated that activated Ras-GTP levels were about fourfold higher in neurofibromas than levels in non-NF1-associated schwannomas.[75,77]

Neurofibromas are most likely clonal tumors derived from a Schwann cell progenitor. The detection of LOH in a tumor operationally defines it as being clonal in origin, and these data are consistent with direct marker analyses in neurofibromas.[57,61] The LOH or other somatic mechanisms that inactivate *NF1* in a Schwann cell progenitor may be an early or initiating genetic event in neurofibromagenesis. Klewe and colleagues showed

LOH in primary neurofibroma tissue and in tumor-derived Schwann cells but not fibroblasts.[78] Other investigators confirmed this observation.[66,68] Two genetically distinct Schwann cell populations, $NF1^{+/-}$ and $NF1^{-/-}$, were successfully cultured from 10 mutation-characterized neurofibromas, whereas tumor-derived fibroblasts carried only the germline $NF1^{+/-}$ genotype.[65] Sherman et al.[79] used an elegant single cell Ras-GTP assay to show elevated levels in neurofibroma-derived Schwann cells but not fibroblasts. Consistent with two Schwann cell populations, only a fraction (12%–62%) of neurofibroma-derived Schwann cells had elevated Ras-GTP levels. The basis of the Schwann cell heterogeneity is not known, but the authors speculate that the cells with high Ras-GTP (presumably neurofibromin-deficient) may recruit Schwann cells with lower Ras-GTP levels (presumably the constitutional neurofibromin-haploinsufficient cells) via the synthesis of growth factors. Whether this admixture of Schwann cells is important in tumorigenesis remains to be determined; however, its observation in primary neurofibroma tissues makes it more likely. Fluorescence in situ hybridization and immunohistochemistry demonstrated that S-100 protein (Schwann cell marker) immunopositive cells in sections of four of seven primary plexiform neurofibromas were monosomic for *NF1*.[80] Because other cells types were disomic at *NF1*, these results strongly implicate the Schwann cell as the target of the *NF1* 2nd hit mutation and verify that the results from neurofibroma-derived Schwann cells were not biased by cell culture. These data also support Schwann cell genetic heterogeneity, because the fraction of S-100 protein positive cells showing *NF1* deletion ranged from 50%–93%. Further evidence for the importance of Schwann cells in neurofibromagenesis comes from the tumorigenic properties exhibited by neurofibroma-derived cells.[81–84] In the most comprehensive studies, Muir and colleagues[83,84] showed that neurofibromin-deficient Schwann cells, derived from either dermal or plexiform neurofibromas, had high invasive potential and produce neurofibroma-like tumors when engrafted into peripheral nerves of *scid* mice.

Germline Alterations That May Modify Neurofibromagenesis

The considerable variable expressivity of NF1 disease among family members with presumably the same *NF1* mutation and results of a statistical trait analysis led to the contention that variation in an individual's genetic background modified the NF1 phenotype.[46,85] To date, no such germline modifying genes have been identified. However, the study of patients carrying *NF1* microdeletions has provided compelling evidence for a gene that modifies neurofibromagenesis. We first recognized that *NF1* microdeletion patients typically showed an early age at onset of localized cutaneous neurofibromas or, in cases in which age at onset could not be documented, they had significantly greater numbers of cutaneous neurofibromas relative to their age.[38,86] It is common to observe multiple cutaneous neurofibromas during physical examination of children under age 10 that are heterozygous for an *NF1* microdeletion,[38,87,88] whereas only about 10% in the general NF1 population have a tumor(s) by that age.[47] In our most severe case, a 5 year old with an *NF1* microdeletion had 51–100 tumors.[38] The *NF1* microdeletions can be inherited from an affected parent, and in these cases, the microdeletion cosegregated with the early age at onset of neurofibromas.[88,89] Other investigators have confirmed the early onset and heavy burden of cutaneous neurofibromas in *NF1* microdeletion patients, and over 50 such patients have been identified.[90–98]

Most *NF1* microdeletions span 1.5 MB of DNA,[87] which harbors the entire 350 kb *NF1* gene and at least 11 additional genes, most of unknown function.[87,99] The microdeletions are preferentially maternal in origin[87,91,93,100,101] and arise by homologous recombination between 60 kb misaligned paralogs (termed NF1REP) that flank the *NF1* gene.[87] Paralogs are nonallelic DNA sequences with a high degree of identity that arose via duplication, e.g., the two functional α globin genes. Over 25 human disorders are known to be caused by gene or chromosomal rearrangements mediated by homologous recombination between paralogs, a process correctly referred to as nonallelic homologous recombination in the review by Stankiewicz and Lupski.[102] Here, I propose that this process be called by the less awkward term of paralogous recombination and that disorders arising by this mechanism be called paralogous recombination disorders, rather than the currently used genomic disorders. Although the NF1REPs share >95% sequence identity over 60 kb of sequence, about 80% of the *NF1* deletion alleles are virtually identical because their breakpoints map to one of either two recombination hotspots of several kb in size located within the NF1REP[103] (M. Dorschner et al., in preparation). Although the molecular basis for these hotspots is not yet known, their existence means that the majority of microdeletion patients will be deleted for the same set of genes. The early age at onset of cutaneous neurofibromas observed in several patients with larger deletions and/or different breakpoints

indicates that generation of a deletion-specific fusion gene product is unlikely.[87]

We hypothesized that the codeletion of *NF1* and an unknown linked gene potentiates cutaneous neurofibromagenesis.[38,87] What might be the function of the putative neurofibromagenesis-potentiating locus (*NPL*)? Here I propose two models for the early age at onset of cutaneous neurofibromas in microdeletion patients. Haploinsufficiency for *NPL* could increase the frequency of somatic 2nd hit mutations in the *NF1* gene. This could result from a genomic instability in microdeletion patients, which is intriguing in view of reports detecting cytogenetic abnormalities and microsatellite instability in some neurofibromas (see below). It would be interesting to determine if somatic *NF1* mutations in cutaneous neurofibromas of microdeletion patients occur by a predominant mechanism. Only a single tumor from each of two deletion patients have been analyzed; one had a 4 bp intragenic deletion[63] and the other a splice site.[65] A second model for early onset of cutaneous neurofibromas proposes that *NPL* haploinsufficiency increases the probability that a neurofibromin-deficient progenitor cell proliferates and manifests as a neurofibroma. Multiple mechanisms could be proposed. For example, *NPL* could encode (or regulate) a cytokine, cell cycle regulator, tumor suppressor, or oncogene, which exerts a positive proliferative advantage on the progenitor cell. Because of the cellular heterogeneity of neurofibromas, this model does not necessarily require that the abnormal expression of *NPL* or its putative downstream targets occur in the neurofibromin-deficient Schwann clone.

Preliminary, but intriguing evidence suggests that genetic background, other than *NF1* microdeletion, may influence the somatic inactivation of *NF1*. In patients with multiple neurofibromas, it was determined that each of the tumors showed the same type of somatic mutation event (e.g., LOH of the entire q arm or interstitial LOH).[56] Depending on the extent of LOH and the particular genes involved, this could explain differences in the age at onset and/or numbers of neurofibromas that develop in an individual.

Somatic Alterations That May Modify Neurofibromagesis

The NF1-associated neurofibromas have been analyzed for genetic abnormalities at the chromosomal level by comparative genome hybridization (CGH) and cytogenetic analyses. Comparative genome hybridization is a powerful technique to detect and map chromosomal regions with copy number imbalances in tumor specimens (reviewed in Ref. [104]). Only two of eight neurofibromas (type not specified) examined showed chromosomal imbalances; one tumor showed three gains; the other only a single gain.[105,106] This observation is consistent with cytogenetic studies. Although Schwann cell cultures from dermal neurofibromas had normal karyotypes, cells derived from plexiform neurofibromas had abnormalities, which in some tumors consisted of unrelated non-clonal abnormalities.[107] One plexiform had structural abnormalities predominantly involving telomeres, which are typically associated with genomic instability in other syndromes/tumors.[107] Wallace et al. proposed that chromosomal abnormalities might be important in the development of plexiform neurofibromas. Chromosomal abnormalities in plexiform neurofibromas may account, at least in part, for their increased risk of malignant transformation. Whether other cellular components of neurofibromas show cytogenetic abnormalities is unclear. Some neurofibroma fibroblast-like derived cultures were reported to show an increased frequency of chromosomal aberrations,[108] whereas others were typically negative.[109]

Conflicting data have been reported regarding the presence of microsatellite instability in NF1-associated neurofibromas. Some human tumors, most notably those of patients with hereditary nonpolyposis colon cancer HNPCC, show microsatellite instability, which is detected by random changes in the length of microsatellite (simple nucleotide repeats) loci. Length mutations at multiple microsatellite loci in a tumor reflect a genome-wide instability, which in the case of HNPCC is due to a defect in any of several mismatch repair genes (reviewed in Ref. [2]). Ottini et al.[110] reported that 50% (n = 16) of neurofibromas showed altered allele lengths compared with matched normal tissue, and instability at chromosome 9 loci has also been reported.[111] Birindelli et al.[112] observed instability in a primary MPNST and a metastasis in one of 25 cases. However, no evidence of instability was detected in two subsequent studies of 80 neurofibromas, of which 5% appear to be of the plexiform type.[55,73] This disparity may be due to technical differences, the number and type of microsatellite loci examined, and/or the stage of the neurofibromas. The LOH at *NF1* was not a factor, because all three studies analyzed neurofibromas that were both positive and negative. Of the eight neurofibromas with microsatellite instability, seven were unstable at only one of the five loci tested.[110] Due to the important implications that microsatellite instability would have for neurofibromagenesis, additional loci should be examined in

these tumors, including those analyzed by the other investigators. In addition, it would be interesting to know if the neurofibromas that showed microsatellite instability were of the larger, central plexiform type, in which case they may have been transforming to malignancy. The proportion of loci that display instability is known to increase with tumor progression in HNPCC.[113]

GENETICS OF MPNST DEVELOPMENT

Homozygous Inactivation at *NF1*

The homozygous inactivation of *NF1* in NF1-associated MPNST, first reported by Skuse et al.,[60] has been confirmed in about 50% of tumors (n = 22) by LOH.[67,109,112,114,115] Mutations in both *NF1* alleles have been identified in a single MPNST.[114] Although the mechanism of LOH is not known, it does not generally involve cytogenetically detectable losses at 17q1.[116] Furthermore, NF1-associated MPNST-derived cell lines showed decreased or absent neurofibromin and high levels of active Ras-GTP,[117,118] and a quantitative Ras activity assay demonstrated that activated Ras-GTP levels in tumors were about 15-fold higher than levels in non-NF1-associated schwannomas.[77] Because homozygous inactivation of *NF1* occurs in benign neurofibromas, it is considered an early or initiating event that is necessary and sufficient for neurofibromagenesis but not MPNST development. Malignant transformation is presumably driven by predisposing genetic factors in the germline and/or by additional somatic mutations and positive growth selection in a malignant precursor cell. The role of *NF1* in the development of sporadic MPNST is not clear; only about 10% of these tumors show LOH at *NF1*.[112]

Germline Alterations That May Modify MPNST Development

Little is known about germline genetic modifiers that predispose to MPNST. Early speculation that patients with *NF1* microdeletion may be at increased risk for malignancy[38] is now supported by indirect evidence. Mutational analysis of germline DNA from seven patients who developed MPNST determined that three (42%) were heterozygous for an *NF1* microdeletion.[119] In another study, 2 of 17 (11%) unrelated *NF1* microdeletion patients developed MPNST, and affected first-degree relatives of two microdeletion patients had other malignancies.[87] Although additional cases are needed, these data suggest that the lifetime risk of MPNST in microdeletion patients may exceed the already high 10% in the general NF1 population.[9] If so, the underlying mechanism may be essentially the same as that proposed above for early onset neurofibromagenesis. Deletion of the putative *NPL* gene could result in genomic instability or exert positive growth selection for the malignant clone. If microdeletions do predispose to both cutaneous neurofibromas and MPNST, it seems reasonable to speculate that in at least this subset of patients, either the discrete cutaneous type of neurofibroma is at increased risk of malignant transformation or the frequency of nodular or diffuse plexiform neurofibromas is high.

Evidence from two families suggests the intriguing possibility that MLH1 deficiency predisposes to NF1 and early onset extracolonic tumors.[120,121] Germline heterozygous inactivating mutations in *MLH1* cause inefficient DNA mismatch repair, with the consequent increase in mutation frequency and susceptibility to hereditary nonpolyposis colorectal cancer (reviewed in Ref. [122]). Two rare and independent cases of consanguineous marriages between *MLH1* heterozygous first cousins each produced two children with NF1 disease and hematological malignancies.[120,121] The parents had no signs of NF1 and there was no family history of the disease. The MLH1 mutations were identified, confirming homozygosity in three of the four deceased children, who presented with mutiple café au lait spots (4/4), dermal neurofibromas (2/4), tibial pseudoarthrosis (1/4), non-Hodgkin's lymphoma (2/4), myeloid leukemia (2/4), and medulloblastoma (1/4). The authors suggest that these patients had a de novo postzygotic *NF1* mutation and that the *NF1* gene may be preferentially susceptible to mismatch repair deficiency. Unfortunately, the *NF1* gene was not analyzed for mutations, due in part to lack of patient tissue,[120] which could have confirmed mutation of *NF1* and differentiated between postzygotic mutation and germline mosaicism in a parent.

Somatic Alterations in NF1-Associated MPNST

All NF1-associated MPNST showed significant chromosomal imbalances by CGH.[105,106,123,124] Analysis of 27 total tumors from 19 NF1 patients showed an average of >7 imbalances per MPNST (Table 1). One tumor had only a single imbalance, a gain of chromosome 8.[123] The studies led by Mechtersheimer and Schmidt,[105,106] which include 74% of tumors examined, detected chromosomal gains more frequently than

Table 1. Chromosomal gains and losses in MPNST detected by CGH.

	NF1-associated MPNST			Sporadic MPNST		
	Loethe[a]	Mechtersheimer[b]	Schmidt[c]	Loethe[a]	Mechtersheimer[b]	Schmidt[c]
No. tumors/No. patients	7/7	6/6	14/6	3/3	13/13	22/20
Imbalances	52	77	188	14	176	200
Per tumor	7.4	11.7	13.4	4.6	13.5	9.1
Range	1–17	6–30	7–29	1–7	0–34	0–25
Chromosome gains	14	48	139	4	125	179
Per tumor	2	8.0	10.0	1.3	9.6	8.1
Range	0–3	4–18	5–20	0–2	0–23	0–21
Chromosome losses	38	29	49	10	51	33
Per tumor	5.4	4.8	3.5	3.3	3.9	1.5
Range	0–14	1–12	0–11	1–4	0–11	0–9

a: Ref. [123].
b: Ref. [105].
c: Ref. [106].

losses (Table 1). Each of the 20 tumors in these studies had ≥ 4 chromosomal gains. Although the results of these two studies were comparable, they differed from a third study that found chromosomal losses more prevalent than gains.[123] The reason for the disparity is unclear; however it was also evident in the analyses of sporadic MPNST in each study (Table 1). Table 2 summarizes the chromosomal segments that most frequently showed gains in NF1-associated MPNST. The most common segments were on 17q22-q24, 17q25, 7p14, 7p21, 8q22, 8q23-q24, and 7q31. Chromosomal loss of these segments was rarely, if ever, observed.[105,106,123] A combined analysis of sporadic and NF1-associated MPNST revealed a significantly decreased survival rate of patients with MPNST with gains at both 7p15-21 and 17q22-qter.[124]

In addition to chromosomal gains, CGH analyses also revealed large-scale chromosomal amplifications.[105,106,124] One-third of MPNST of both NF1 and non-NF1 patients had at least one amplified chromosomal segment (Table 3). Although the number of tumors is small, there are differences in amplification patterns. In NF1-associated MPNST 7p and 17q22-qter, the same regions that commonly showed chromosomal

Table 2. Frequency of chromosomal gains in NF1-associated MPNST.

	% MPNST			
	Loethe[a]	Mechtersheimer[b]	Schmidt[c]	Total
No. MPNST/No. patients	7/7	6/6	14/6	27/19
Chromosome				
1q33	0	50	50	37
5p15	28	50	35	37
7p14	28	83	71	63
7p21	28	50	64	52
7q31	28	33	64	48
8q22	28	33	64	48
8q23-q24	14	33	85	55
15q24-q25	0	17	71	41
17q22-q24	42	83	85	74
17q25	71	83	78	78

a: Refs. [105,123].
b: Ref. [105].
c: Ref. [106].

gains (Table 1) were amplified frequently. The frequency of 7p amplifications may be overestimated because these four MPNST (considered as different primary tumors) were from a single patient.[106,124] In contrast, amplifications of 5p, 8q, and 12q were the most prevalent in sporadic MPNST. There was a significant difference in the frequency of tumors with more than one chromosomal segment amplified. Only 10% of NF1-associated MPNST had more than one amplified segment, whereas the frequency was 72% in sporadic tumors (Table 3). Differences in the location and number of chromosomal segments amplified were unlikely to be due to tumor grade, because the majority of MPNST, 14 of 20 (70%) of NF1-associated and 22 of 34 (64%) of sporadic, were grade 3 (poorly differentiated). Although amplifications were more frequent in NF1-associated than sporadic MPNST (50% vs. 32%), the number of NF1-associated tumors may be overestimated because it includes multiple tumors from the same patient. Adjustment for one tumor/patient gives a frequency of 25% (5 of 20) for NF1-associated MPNST. Similarly, there was no correlation between chromosomal

Table 3. Large-scale chromosomal amplifications in MPNST.[a]

Chromosome segment amplified	No. NF1-associated MPNST[b] (N = 20)	No. sporadic MPNST[c] (N = 34)
4q12-q13		1
5p11-p15		1
5p14		1
5p15		2
5p13-pter		2
7p14-pter	2[d]	1
7p13-pter	1[d]	
7p12-pter	1[d]	
7p11-p12		1
8q12-qter		2
8q13		1
8q21-q22		1
8q22-q23		1
8q24		1
8q23-qter	1	
9p21-p23	1[e]	
9q31-q33		1
12p12	1[e]	
12p13		1
12q13-q14		1
12q14-q21		3
12q24		3
13q13-q33		1
13q32-q33		1
17p11-p12		1
17q24-qter	2	
17q22-q24	1	
20q12-qter		1
Xp11		1
Xp21-p22		1
Summary		
% tumors with an amplification	50 (10/20)	32 (11/34)
% tumors with >1 amplified segment	0.1 (1/10)	72 (8/11)
% patients with $\geq$1 tumor with an amplification	30 (4/12)	32 (11/34)

a: Centromeric regions, chromosomes 19 and Y, and 1p32-p36 were not scored for technical reasons. (From Ref. [105].)
b: Refs. [105,106,124].
c: Refs. [105,124].
d: Four of five primary MPNST from one patient. (From Ref. [106].)
e: Two of four primary MPNST from one patient. (From Ref. [106].)

amplification and progression to metastasis. Analysis of tissue from a primary tumor, recurrence, and metastasis from a single patient showed a single amplification (8q12-qter) in the recurrent tumor.[124]

Comparison of the chromosomal losses detected in the three CGH studies revealed the following five most frequent losses in 27 NF1-associated MPNST: 13q21-22 (12/27), 11q23-25 (10/27), 1p22-31 (9/27), 3q11-21 (8/27), and 17p12-pter (7/27).[105,106,123] It is of interest that the loss of 17p12-pter was detected in 50% (7 of 14) tumors in one study[106] and in none of the tumors of the other studies. The relatively low detection rate of chromosomal losses may be due to the decreased sensitivity of CGH in polyploid MPNST.[125]

The CGH analysis of multiple, presumably synchronous or metachronus, primary MPNST at different sites in three NF1 patients revealed a remarkable similarity in chromosomal gains and losses.[106,124] For example, five grade 3 (poorly differentiated) tumors of one patient each showed imbalances of +7p, −13q21, and +17q22-qter. These data showed that in the specific genetic background of each patient, a relatively limited, and defined, number of rearrangements were shared among the tumors. Similarly, nearly identical aberrations were found in different MPNST from the same patient.[125] Although limited, these data suggest that each individual's constitutional genotype sets a certain "baseline" on which a minimal and limited number of genetic alterations are necessary and sufficient for MPNST development.

Consistent with CGH analysis, the karyotype of NF1-associated MPNST-derived cells are complex with chromosomal numbers ranging from 34 to 270 indicative of hypodiploidy, hypotriploidy, hypotetraploidy, hypertriploidy, and hypertetraploidy (Refs. [109,116,125] and references therein). Although breakpoints were frequent, a common specific breakpoint was not detected. A comparison of CGH and karyotying in six MPNST revealed significant overlap in the most frequent gains and detected losses at 19q (3 of 6 tumors), a region not analyzed in the CGH studies.[106,125] Plaat et al.[116] performed a computer-assisted cytogenetic analysis of 46 MPNST reported in the literature and 7 new cases of both NF1-associated and sporadic tumors (Ref. [116] and references therein). These studies confirmed the CGH observation of high-frequency gains at chromosomes 7p and 7q and losses at 1p and 17p. However, their reported cytogenetic differences between NF1-associated and sporadic MPNST[116] were not confirmed in later studies.[125,126] In one study, near triploid or near tetraploid clones were associated with grade 3 tumors and a poor prognosis.[126] The detection of a t(X;18) translocation in MPNST[127] was not confirmed in either sporadic[128] or NF1-associated neurofibroma or MPNST.[129]

Inactivating mutations in several tumor suppressor genes have been identified in NF1-associated MPNST. Both LOH and intragenic missense mutations of *TP53*[50,62,112,130] have been detected. Like many sarcomas, NF1-associated MPNST often showed overexpression of p53 (the protein product of the *TP53* gene) as assayed by immunoreactive positivity in the nucleus.[50,112,131,132] In keeping with findings in other tumors, this most likely is mutant p53 protein, which accumulates due to its increased stability. Mutant protein is thought to promote cancer by either complexing with and sequestering functional p53 or by complexing with p63 and p73 and blocking their normal transcription factor activities (reviewed in Ref. [133]). Immunohistochemical detection of p53 was more common in NF1-associated vs. sporadic MPNST, and it was associated with poor prognosis in NF1 children.[132] About 50% of NF1-associated MPNST showed homozygous deletion for exon 2 of the *INK4A* gene, and over 90% were immunonegative for its protein product p16.[112,134,135] Homozygous deletion of exon 2 results in deficiency for both p16 and $p14^{ARF}$, two proteins encoded by alternative splicing of *INK4A* (also known as *CDKN2A*). Both of these proteins are tumor suppressors that modulate activities of the RB and p53 pathways, which are critical for cell cycle control and tumor surveillance (reviewed in Ref. [136]). MXI1 mutations in regions that encode known functional domains have been detected in the two NF1-associated MPNST analyzed.[137] Mxi1 is an agonist of the oncoprotein Myc and is thought to limit cell proliferation and help maintain the differentiated state (reviewed in Ref. [138]). *Mxi1*-deficient mice develop tumors, and *Mxi1* deficiency decreases the latency of tumors that arise in Ink4a-deficient mice.[139] If additional studies show that somatic inactivation of MXI1 is common, it would suggest a link between the pathways of Ink4a, Myc, and Ras in NF1-associated MPNST.[140,141]

Several differences observed in NF1-associated MPNST are likely involved in the malignant transformation of a preexisting neurofibroma. *TP53* or *INK4A* mutations/altered expression were not detected in neurofibromas.[50,51,54,62,67,69,112,132,134,135] Furthermore, p53-positive nuclei, typically associated with MPNST, were observed in a few cells at the transitional zone between an existing plexiform neurofibroma and an arising MPNST.[51] One plexiform neurofibroma proximal to an MPNST did show p16 immunonegativity.[112] Microdissection of a preexisting neurofibroma from its MPNST focal malignant process revealed 5 chromosomal imbalances in the neurofibroma, all of

which were novel compared to the 10 imbalances in the MPNST component.[106] These data are consistent with a restructuring of the genome during transformation. An additional distinguishing feature of MPNST is the high labeling index of the nuclear proliferating antigen Ki67, which was correlated with reduced survival in a study that combined NF1-associated and sporadic MPNST.[51,132,142]

OTHER NF1-ASSOCIATED NEOPLASMS AND MOUSE MODELS

In addition to peripheral nerve sheath tumors, individuals affected with NF1 are at increased risk for an array of other tumors. Epidemiologically associated neoplasms include medulloblastoma, pheochromocytoma, astrocytoma, and adenocarcinoma of the ampulla of Vater (Ref. [143] and references therein).[144] Primarily children affected with NF1 are at increased risk for optic pathway gliomas and brainstem gliomas, rhabdomyosarcomas, and malignant myeloid leukemias.[41,145–149] The NF1 patients are also at increased risk for a second malignancy, some of which may be treatment-related.[146,150,151] In different studies, 8%–21% of NF1 patients with a first malignancy developed a second cancer, compared to a frequency of 4% in the general population.[143] Malignancy in NF1 has been reviewed recently.[152]

Although heterozygous mutant mice ($Nf1^{+/-}$) develop tumors, they are not the characteristic peripheral nerve sheath tumors characteristic of the human disease.[153–155] The *Nf1*-deficient mice ($Nf1^{-/-}$) die in utero from cardiac defects. Mice chimeric for ($Nf1^{-/-}$) and ($Nf1^{+/+}$) cells were able to develop many microscopic plexiform neurofibromas, but dermal tumors did not develop.[156] In addition, mice doubly heterozygous for mutations in *Nf1* and *p53* developed MPNST that showed LOH at both tumor suppressor loci.[156,157] Recently, mice were constructed such that only their Schwann cells were *Nf1* deficient.[158] Different tumor phenotypes were observed, depending on whether the *Nf1*–deficient Schwann cells were in an animal with an $Nf1^{+/-}$ or an $Nf1^{+/+}$ constitutional genotype. In the $Nf1^{+/-}$ genetic background, plexiform neurofibromas composed of *Nf1*–deficient Schwann cells, and the fibroblasts and mast cells that normally occur in human neurofibromas, developed on peripheral nerves. In the $Nf1^{+/+}$ genetic background, *Nf1*–deficient Schwann cells did not participate in neurofibromagenesis but did form relatively small hyperplastic lesions of the cranial nerves containing minimal mast cells. This mouse model demonstrates that neurofibromin deficiency in Schwann cells is sufficient for generating nascent tumor lesions, but frank plexiform neurofibroma development requires neurofibromin haploinsufficiency in cells of the surrounding tissues.

With the development of mouse models, understanding the genetics, pathology, and natural history of human benign and malignant peripheral nerve sheath tumors takes on new importance. It is only by accurate modeling of the human disease that progress can be made toward therapies that can slow or halt neurofibromagenesis and reduce the high risk of malignancy associated with NF1 disease.

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